# REPROGRAMMING OF HUMAN STEM CELLS TO HEPATOCYTES FOR CLINICAL AND INDUSTRIAL APPLICATIONS

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**Abstract**

The aim of this research project is to reprogramme human stem cells into hepatocytes in a robust and scalable manner for clinical and industrial applications. Hepatocytes are the parenchymal cells that make up the majority of the liver. The shortage of hepatocytes and difficulty in maintaining primary hepatocytes will remain key obstacles that must be overcome by researchers before hepatocyte transplantation can be used in clinical trials.

Stem cells possess the unique ability to differentiate into any cell type in our body. In addition, they possess the ability to self-renew, meaning, they can act as a renewable source of hepatocytes. Thus, the goal is to differentiate human embryonic stem cells (hES) to hepatocyes using cytokines and growth factors (GFs). Cytokines and growth factors were used in the past to mimic embryonic liver development, thus, the goal of this project is to advance a step further and to mimic adult liver development. The difficulty in this system is to translate adherent stem cell culture to a suspension culture that better suits their optimal culture environment.

In this project, the focus will primarily be on the differentiation of human stem cells to hepatocytes. After rigorous cell culturing and data collection, it has been found that on day 10 of differentiation, the cells were well under their way of developing as hepatocytes, however they did not show target results for each gene expression tested for.

**Introduction**

In order to understand the background and technical aspect that this particular research project will be undertaking, it is important to understand the current status of the field of stem cell technology.

In addition to the clinical aspects of stem cell-derived hepatocytes, these cells can be used in the industrial setting for metabolic studies or further toxicity testing to improve drug efficacy[1]. Hepatocytes are especially attractive candidates for drug toxicity studies because of their active metabolic pathways and signalling during drug metabolism[2]. The goal of using stem cell-derived hepatocytes would be to use them as an alternative source instead of animal trials to better estimate acute and chronic toxicity from certain drugs[3]. The shortage of human livers makes it extremely difficult to use primary hepatocytes for pharmaceutical purposes prompting the need for an alternative source[4].

This need to develop hepatocytes on a larger scale is not limited to the research and manufacturing of drugs. Instead, their use may possibly treat chronic liver diseases that impact "over 30 million Americans and over 600 million people"[5] all over the world. However, "alternative tissue sources such as fetal hepatocytes and hepatic cell lines are unreliable, difficult to reproduce, [as they] do not fully recapitulate hepatocyte phenotype and functions"[6], and therefore, must be replaced by an alternative source.

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[1] Li, Albert P. *Human Hepatocytes: Isolation, Cryopreservation and Applications in Drug Development*. N.p., 9 Jan. 2007. Web. 20 Sept. 2014.
[2] *Ibid.*
[3] JV et al. "Hepatocyte Cell Lines: Their Use, Scope and Limitations in Drug Metabolism Studies." *National Center for Biotechnology Information*. US National Library of Medicine National Institutes of Health, Apr. 2006. Web. 20 Sept. 2014.
[4] Si-Tayeb et al. "Highly Efficient Generation of Human Hepatocyte–like Cells From Induced Pluripotent Stem Cells." *Wiley Online Library*. American Association for the Study of Liver Diseases, 1 Oct. 2009. Web. 20 Sept. 2014.
[5] Schwartz et al. *Pluripotent Stem Cell-Derived Hepatocyte-like Cells*. *Biotechnology Advances*. Elsevier, 16 Jan. 2014. Web. 20 Sept. 2014.
[6] *Ibid.*

Researchers have looked into using "human embryonic stem (hES) cell and induced pluripotent stem (iPS) cells"[7] as 'alternative cell sources' as hES cells provide a renewable cell source of functional human hepatocytes[8]. The ultimate end goal would be to use these differentiated hepatocytes towards more advanced and ambitious research such as hepatocyte transplantation and drug toxicity testing.

Current state of the art cultivation of stem cells involves 3 dimensional culture of stem cells and co-culture with non-parenchymal cells (NPCs) in order to mimic the native microenvironment[9]. These attempts are techniques researchers have used in order to further mature stem-cell derived hepatocytes.

Current differentiation methods result in iPS-derived hepatocyte-like cells or ES-derived hepatocyte-like cells possessing some functional hepatic activity similar to primary hepatocytes. Stem cell-derived hepatocytes secrete urea, alpha-1-antitrypsin, and albumin[10], revealing that the combined phenotypic and functional traits exhibited by ES-derived hepatocytes resemble a relatively immature hepatic phenotype that more closely resembles that of fetal hepatocytes rather than adult hepatocytes[11].

Therefore, "theoretically, for a cell to become a terminally differentiated cell type, it must undergo all consecutive steps of development to be able to respond to the surrounding differentiation cues"[12] in order to mature into a successful adult hepatocyte. Thus, keeping this

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[7] *Ibid.*
[8] Rambhatla et al. "Generation of Hepatocyte-like Cells From Human Embryonic Stem Cells." *Cell Transplantation*. Ingenta Connect, 1 Nov. 2003. Web. 20 Sept. 2014.
[9] Godoy et al. *Recent Advances in 2D and 3D in Vitro Systems Using Primary Hepatocytes, Alternative Hepatocyte Sources and Non-Parenchymal Liver Cells and Their Use in Investigating Mechanisms of Hepatotoxicity, Cell Signaling and ADME*. Springerlink, 23 Aug. 2013. Web. 20 Sept. 2014.
[10] Schwartz et al. *Pluripotent Stem Cell-Derived Hepatocyte-like Cells*. *Biotechnology Advances*. Elsevier, 16 Jan. 2014. Web. 20 Sept. 2014.
[11] *Ibid.*
[12] Roelandt et al. *Human Embryonic and Rat Adult Stem Cells with Primitive Endoderm-Like Phenotype Can Be Fated to Definitive Endoderm, and Finally Hepatocyte-like Cells*. N.p., 11 Aug. 2010. Web. 20 Sept. 2014.

end result of a mature hepatocyte in mind, this research project will be based upon the imitation of the step-by-step process which is followed by the hepatocytes in vivo and will be reconstructed in vitro.

This research project will then contribute to the already existent researches as it would verify the practicality of the aforementioned theoretical process on the development of mature hepatocytes through the imitation of the step-by-step biological process occurring in vivo. Thus, the objective of this research is to develop a robust process of producing stem cell-derived hepatocytes.

**Materials and Method**

In this project, various materials were used, and the methods that were used in this project have been described below.

Since human stem cells were used in this project, it was important to grow them in a medium to maintain their pluripotent state and inductive of growth. Therefore, this project used basic Fibroblast Growth Factor (FGF) in order to maintain the pluripotency of the stem cells as demonstrated by a number of research groups. Cells were then grown in a high glucose DMEM solution to provide the adequate nutrient and metabolites for optimal growth and survival of the cells.

Once the stem cells were confluent and ready for expansion in a feeder environment, they were passaged in order to increase the surface area on which the cells could attach, thereby increasing the number of hES cells. Once adequate amount of cells were cultures, the process of differentiation was initiated. It is a 20-day procedure in which stem cell samples on Days 0, 6, 10, 14 and 20 are collected for analysis.

Differentiation was done in 12 well plates (Corning 12 wells 3513) which were then coated with 2% Matrigel (BD 356231) and diluted in PBS solution (Gibco 10010). These

wells were then kept for 1 hour in a 21% $O_2$ – 5.8% $CO_2$ - 37°C incubator. Once the hES were transferred from 6 well plates onto the aforementioned 12 well plates, 4 different stage media were used in order to initiate the differentiation process[13].

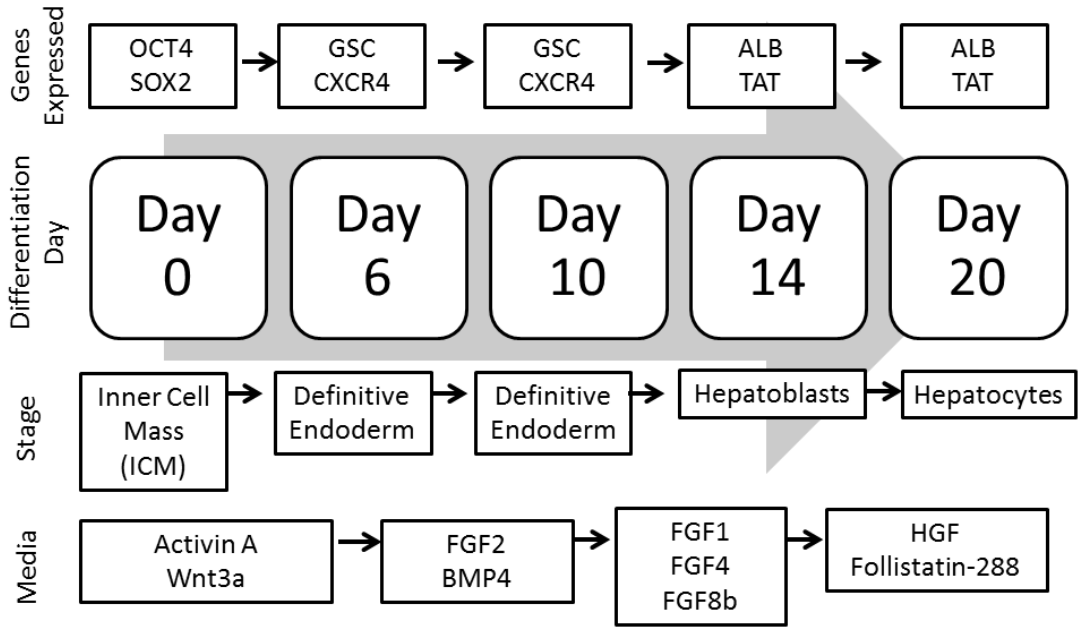


Figure 1.0: Diagram showing the progression of the differentiation procedure for hepatocytes

Once the differentiation procedure was completed, it was important to test whether the cells resembled primary hepatocytes in the body. For this step, it was necessary to carry out quantitative reverse transcription polymerase chain reaction (RT-qPCR). Therefore, a commercially available RNeasy mini kit / micro – kit (Quagen 74104 and 74004) was used to carry out RNA isolation. The next step was to synthesise cDNA, for which 1 µg of RNA with Superscript III First-Strand synthesis system (Invitrogen 11733-046) was used. Finally, SYBR Green Platinum SYBR green qPCR Supermix-UDG (Invitrogen 11733-046) was used to

[13] See Figure 1.0

perform real time PCR. Once the gene levels were measured, the relative expression of every gene was calculated with respect to GAPDH expression[14].

**Results**

| Differentiation Day | GAPDH | OCT4 | SOX2 | CXCR4 | ALB | TAT | GSC |
|---|---|---|---|---|---|---|---|
| 0 | 22.19 | 37.39 | 24.58 | 36.7 | - | - | - |
| 10 | 18.77 | - | 23.56 | 30.01 | 34.71 | 35.44 | 36.27 |

Figure 2.0: Table showing cycle number at which the respective genes were expressed

| Differentiation Day | OCT4 | SOX2 | CXCR4 | ALB | TAT | GSC |
|---|---|---|---|---|---|---|
| 0 | 3.27 | -3.05 | 14.51 | - | 9.43 | 7.24 |
| 10 | 21.27 | 6.75 | 6.95 | 18.56 | 17.52 | 17.16 |

Figure 3.0: Table showing the performance of each gene with respect to the Gapdh gene (ΔCT)

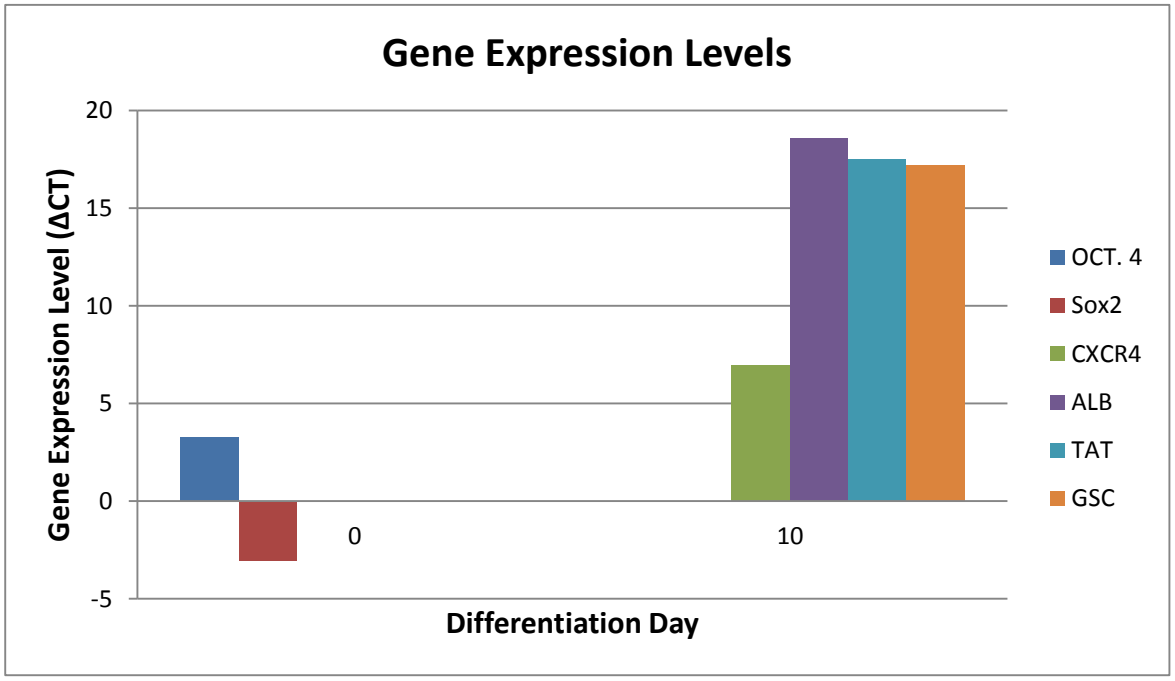


Figure 4.0: Graph showing the gene expression levels in hepatocyte-like cells

[14] See Figures 2.0, 3.0 and 4.0

**Discussion**

The results for PCR after differentiation should show different trends for different genes. For example, genes Oct 4 and Sox2 should have a ΔCT ranging from negative numbers to 3 for Day 0 of differentiation, CXCR4 and GSC should have a ΔCT ranging from 4 to 10 for Day 10 samples and ALB and TAT (liver markers) should range from 10 to 15 on Day 10 and 14 of differentiation. Therefore, a negative number / small positive value of ΔCT means high expression of that gene whereas a high positive value of ΔCT means low gene expression.

Therefore, ideally, Oct 4 and Sox 2 should have a high expression at the beginning of differentiation and low differentiation after differentiation of the cells has occurred. CXCR4 and GSC should show medium expression around Day 10 of differentiation, and finally the liver markers, i.e. ALB and TAT should have low gene expression at the beginning of differentiation and a high expression level near the end of differentiation.

In this project, Oct 4 and Sox 2 show results on Day 0 as expected, CXCR4 and GSC show expression very close to the desired expression levels on Day 10 and finally, ALB and TAT show a fair amount of expression on Day 10.

Therefore, we can infer from the above data that the differentiation began without much hindrance, which is why the data for Day 0 is as expected. However, it is quite possible that the project ran into a few obstacles which is why the differentiation deviated from the right track in the middle of the differentiation, i.e. Day 10.

A possible explanation for these unexpected results during and near the end of differentiation is the utilisation of a not-well differentiated cell culture well. There could be several reasons for why the cells in this particular well were not differentiating properly. For example, the cell media for each stage of differentiation could be flawed, or even before that, while the hES cells were attached onto the Mouse Embryonic Fibroblast (MEF) cells, the H9

media could have been partially differentiated to begin with. In addition, assuming all went well before the differentiation, it is quite possible that some of the primers failed to work properly which is why the data is not cohesive.

Thus, in order to reduce the possibility of incorrect differentiation at any given stage of the project, it is highly recommended to perform PCR on Day 0 of differentiation, as a flawed Day 0 PCR result will be indicative of a flawed Day 20 hepatocyte result in the future.

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